Mutations in the trfA Replication Gene of the Broad-Host-Range Plasmid RK2 Result in Elevated Plasmid Copy Numbers

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Mutated forms of trfA, the replication protein gene of plasmid RK2, that support a minimal RK2 origin plasmid in Escherichia coli at copy numbers up to 23-fold higher than normal have been isolated. Six such high-copy-number (copy-up) mutations were mapped and sequenced. In each case, a single base transition led to an amino acid substitution in the TrfA protein primary sequence. The six mutations affected different residues of the protein and were located within a 69-base-pair region encoding 24 amino acids. Dominance tests showed that each of the mutants can be suppressed by wild-type trfA in trans, but suppression is highly dependent on the amount of wild-type protein produced. Excess mutant TrfA protein provided in trans significantly increased the copy number of RK2 and other self-replicating derivatives of RK2 that contain a wild-type trfA gene. These observations suggest that the mutations affect a regulatory activity of the TrfA replication protein that is a key factor in the control of initiation of RK2 replication.

Control of the frequency of initiation of the broad-host-range plasmid RK2 is not well understood. Replication is known to be initiated at a single origin, termed oriV (11, 36, 39, 42, 43). The only other plasmid locus essential for replication is the trans-acting gene trfA (11, 39, 42). This gene encodes two proteins, termed TrfA-43 and TrfA-32, at least one of which is essential for replication in all hosts (9, 16, 26, 30, 33). It has been proposed that RK2 copy number is determined primarily by regulating the synthesis of the TrfA proteins such that these proteins are present in rate-limiting amounts. Several studies have provided evidence consistent with this hypothesis (25, 28, 41). However, it is clear that there is a mechanism capable of controlling RK2 replication that is independent of TrfA concentration. In the accompanying paper (10), raising TrfA protein levels to two or three times above the normal cell level in Escherichia coli was shown to increase RK2 copy number by only 30%. Further increases in TrfA concentration (up to 170-fold) had no additional effect on plasmid copy number, indicating that TrfA concentration is not the sole determinant of RK2 replication frequency. It was also found (10) that a plasmid containing only the RK2 origin of replication and two antibiotic resistance genes exhibited copy numbers similar to those of RK2 itself in response to various TrfA protein concentrations, suggesting that the TrfA-concentration-independent control mechanism is a function of the properties of the RK2 oriV region, the TrfA proteins, or both.

In order to study the role of TrfA proteins in replication and copy control, the minimal trfA coding sequence was cloned and mutagenized. Mutants were isolated that support oriV at substantially elevated copy numbers compared with those of the wild-type gene. The properties of these mutants suggest that it is critical to copy control both in minimal replicons and in whole RK2. TrfA may, therefore, be a bifunctional protein that acts as both an initiator and a negative regulator of plasmid replication initiation.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. The trfA fragment in pRD110-34 (Fig. 1) was derived from pCT88A34. This plasmid was partially digested with HaeIII and treated with T4 DNA polymerase to blunt the ends. PstI linkers (CCTG CAGG; New England Biolabs) were added to the ends, and the resulting DNA was digested with PstI and EcoRI. This mixture of fragments was added to EcoRI- and PstI-digested pBR322 and treated with T4 DNA ligase. Transformants were selected for tetracycline resistance in E. coli, pooled, and retransformed with the oriV plasmid pCT45. Only those cells that contained a derivative of pBR322 carrying trfA in place of the bla gene were able to support pCT45. Individual transformants were analyzed to identify a plasmid containing the smallest functional trfA fragment, corresponding to nucleotides 408 to 1618 of the trfA sequence (32). This plasmid was designated pRD110-34. A similar scheme was used to construct pRD110-16 from pCT88A16 (Fig. 1). The 3' end of the trfA gene is the same in both plasmids, but pRD110-16 lacks 265 base pairs (bp) of trfA sequence at the 5' end of the gene. As a result, pRD110-34 encodes TrfA-32 and TrfA-43, while pRD110-16 encodes only TrfA-32. The native trfA promoter is absent in each of these constructs, but transcription from the pBR322 anti-tet promoter (37) appears to be sufficient to drive the synthesis of TrfA, as evidenced by the ability of both pRD110-16 and pRD110-34 to support replication of pCT45.

pRD119-34 and pRD119-16 (Fig. 1) were derived from pRD110-34 and pRD110-16 as follows. The 793-bp HindIII-to-BamHI fragment containing the promoterless chloramphenicol resistance gene of pUC8-CAT was filled in at the HindIII site and inserted into the EcoRV and BamHI sites of each of the two pRD110 plasmids. This inactivated the tet gene and placed cat under the transcriptional control of the tet promoter. The same procedure was used to convert pRD110trfAcop plasmids to pRD119trfAcop plasmids. Plas-
mid pSV16 (Fig. 2) contains the EcoRI-to-BamHI oriV fragment of pTJS65 (26) coupled to a BamHI fragment containing the kan gene of pUC5 (45) and an EcoRI-to-BamHI fragment containing the bla gene derived from pBR322 (bp 3104 to 4363) (38). Plasmid pFF1 (Fig. 2) is a self-replicating RK2 derivative containing the BclI-to-EcoRI oriV fragment of pRK248 (42). The trfA gene in pFF1 is derived from pRD110-34. Transcription of the trfA gene is provided by a 354-bp EcoRI fragment containing the neo promoter of Tn5 (bp 1196 to 1519) (4). A 270-bp fragment containing the minimal RK2 origin of transfer (13, 14) is also present. No other RK2 loci are present in pFF1. The bla and cat resistance markers were derived from pBR322 (5) and pUC9Cm, respectively, (K. Buckley, Ph.D. thesis, University of California, San Diego, La Jolla, Calif., 1986).

**Mutagenesis of trfA.** Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was carried out essentially by the method of Miller (19). Five-milliliter overnight cultures of E. coli TB1 containing pRD110-34 or pRD110-16 were grown at 37°C in LB containing 25 μg of tetracycline per ml. Each culture was diluted 1:20 into two separate flasks of the same medium and shaken at 37°C for 1.5 h. The cells were centrifuged, washed twice with 10 ml of citrate buffer (19), and suspended in 25 ml of the same buffer. A 1.25-ml portion of 1-mg/ml NTG in citrate buffer was added to each sample, and the cells were incubated for 20 or 40 min at 37°C. After incubation with NTG, the cells were washed with 10 ml of phosphate buffer (19) and suspended in 10 ml of LB without antibiotic. The cultures were incubated at 37°C for 1.5 h, and then each was added to a 2.8-liter fluted flask containing 250 ml of LB with 25 μg of tetracycline per ml. These were shaken overnight at 37°C.

Plasmid DNA was isolated from an alkaline lystate of each 250-ml culture by CsCl density centrifugation. A 10-μg sample of each DNA was digested with EcoRI and PsI, and the trfA fragment was agarose gel purified. Approximately 2 μg of each of the isolated fragments was mixed with 1 μg of pBR322 that had been previously digested with EcoRI, PsI, and Scal (to inactivate the pBR322 bla gene). After ligation, the subcloned plasmids were transformed into E. coli TB1, yielding 30,000 to 100,000 colonies for each of the four preparations. These colonies were pooled to form the four mutant banks (i.e., two time points of mutagenesis for each of the two plRD110 derivatives).

**Mapping and DNA sequencing of cop mutations.** Digestion of pRD110-16, or its copy-up mutant derivatives, with HincII yielded 3.6- and 0.95-kilobase (kb) fragments, while NdeI digestion yielded 2.95- and 1.6-kb fragments. HincII digestion of pRD110-34 or its derivatives yielded 3.6- and 1.2-kb fragments, while NdeI digestion yielded 3.2- and 1.6-kb fragments. These two enzymes were chosen because they cleave pRD110 once in the vector sequence and once in the trfA gene (Fig. 1). The fragments were separated by electrophoresis on a 0.7% agarose gel, and each was isolated separately by electroelution.

To map a particular mutation pairwise, combinations of purified fragments were ligated together such that a pRD110-type plasmid would be reconstructed, i.e., the 3.6-kb HincII fragment from the wild-type plasmid was ligated to the small HincII fragment from the mutant and vice versa, and similarly for the NdeI fragments. Ligation mixtures were then used to transform competent E. coli HB101 cells to Tc'. Since one of the HincII sites is in the gene encoding Tc' and one of the NdeI sites is in the vector ori, the only ligation products which should give transformants are those in which the two different HincII fragments or the two different NdeI fragments are present and oriented as they are in pRD110.

If the phenotype of each of the copy-up trfA genes was due to a single mutation, then for each enzyme one combination of fragments should give a plasmid with a wild-type phenotype, while the reverse combination would give a plasmid with a copy-up phenotype. The latter would localize the mutation to a particular restriction fragment. The mutations mapped in this way were cop271C and cop254D (using pRD110-16 as the wild-type plasmid) and cop273C, cop267L, cop251M, and cop250V (with pRD110-34 as the wild-type plasmid).

Typically, three HB101 transformants of a given reconstructed plasmid were tested for their abilities to support the origin plasmid, pSV16, at a wild-type or elevated copy number as detected by relative penicillin resistance levels or

### TABLE 1. Strains and plasmids used

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<tr>
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<td>pRD110-16 with cat inserted into tet; Cam’</td>
<td>This work</td>
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<td>pRD110-34 with cat inserted into tet; Cam’</td>
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<td>Naturally occurring plasmid; Pen’ Kan’</td>
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Typically, three HB101 transformants of a given reconstructed plasmid were tested for their abilities to support the origin plasmid, pSV16, at a wild-type or elevated copy number as detected by relative penicillin resistance levels or
as visualized on agarose gels. The results obtained were consistent with all six mutations mapping within the 379-bp HincII-Ndel fragment. This fragment from each of the mutants was then sequenced.

Plasmids for sequencing were constructed by isolating the 379-bp HincII-Ndel fragment from each mutant pRD110 plasmid, filling in the Ndel site by using Klenow polymerase as described previously (17), and then ligating the fragment into HincII-digested pBluescriptSK+ (Stratagene Cloning Systems, San Diego, Calif.). Sequencing plasmids with the insert in either orientation were obtained for each mutant. Single-stranded template DNA was prepared in accordance with instructions supplied with the vector. DNA sequencing was performed by using the Sequenase kit purchased from U.S. Biochemical Corp. The labeling reactions were separated on 8% denaturing polyacrylamide wedge gels.

Other methods. Estimations of TrfA levels by Western blots (immunoblots) were performed as described in the accompanying paper (10). Plasmid copy numbers were determined by the dot blot procedure of Shields et al. (29), modified as described in the accompanying paper (10). NTG was obtained from K&K Laboratories, Hollywood, Calif. Antibiotics were obtained from Sigma Chemical Co., except tetracycline, which was obtained from CalBiochem. Bacterial transformation (8), DNA isolation (2), and gel purification (17) were done as described previously.

RESULTS

Mutagenesis of the trfA gene. Figure 1 shows the structures of plasmids pRD110-16 and pRD110-34, which were used for the mutagenesis of the trfA gene. These plasmids are derivatives of pBR322 in which the EcoRI-to-PstI fragment containing the 5' end of the bla gene has been replaced with a fragment carrying trfA. The trfA fragment in pRD110-34 includes bp 408 to 1618, while the fragment in pRD110-16 includes bp 673 to 1618 (numbering in accordance with the system in reference 32). The trfA gene normally encodes two proteins, called TrfA-43 and TrfA-32, because of the presence of two in-frame initiation codons (16, 30, 33). pRD110-34 retains this property, but pRD110-16 lacks sequences necessary for synthesis of TrfA-43 and specifies only TrfA-32. Transcription of trfA is most likely directed by the anti-tet promoter (designated P1 in reference 37) that is located just clockwise of the HindIII site in the pBR322 vector sequence. An important feature of the pRD110 construct is that trfA expression and activity appear to have little or no effect on the replication of the pBR322 origin of the plasmid. This allowed the generation of mutant banks without imposing any initial selection for trfA phenotypes.

Mutagenesis was performed by treating E. coli TB1 containing either pRD110-16 or pRD110-34 with NTG for 20 and 40 min as described in Materials and Methods. After being

FIG. 1. Maps of plasmids containing wild-type and mutant trfA genes. All plasmids are derived from pBR322 and differ only in antibiotic resistance and whether or not they encode TrfA-43 in addition to TrfA-32, as indicated. The trfA gene (shaded) lacks its native promoter and is most likely transcribed by the promoter labeled Panti-tet. This promoter has also been called P1 (37). Antibiotic resistance genes are tet (tetracycline) and cat (chloramphenicol). kD, Kilodalton.
The purified procedures other systems reduce and grown into the kan, chloramphenicol resistance (bla, Ph.D. thesis); bla, the penicillin resistance gene from pBR322; and kan, the kanamycin resistance gene from pUC5 (45).

washed, the surviving cells were diluted into fresh medium and grown overnight. Plasmid DNA was isolated, and the EcoRI-to-PstI trfA fragment from each preparation was gel purified. The mutagenized trfA preparations were recloned into the EcoRI and PstI sites of pBR322 to recreate pRD110-16 and pRD110-34, respectively. This step was included to reduce the frequency of mutations in the pBR322 vector sequences that might complicate subsequent analysis. These procedures yielded four independent mutant banks (two for each plasmid) in which the trfA gene had been treated with NTG for 20 or 40 min.

Selecting copy-up mutations in trfA. Advantage was taken of the fact that resistance to β-lactam antibiotics is generally proportional to gene dosage. Thus, an increase in the copy number of a plasmid carrying the bla gene leads to a proportional increase in penicillin resistance (44). This fact has been used previously to select copy-up mutations in other systems (20, 27). In this case, the RK2 origin plasmid pSV16 (oriV bla kan) was used to select for mutations in trfA leading to higher oriV copy numbers and increased penicillin resistance. E. coli TB1 carrying pRD110-16 or pRD110-34 (wild type) was transformed with pSV16 DNA and plated on LB containing different levels of penicillin G. In each case, transformants grew well at 250 μg of penicillin per ml, but frequencies of transformation were reduced 10- to 20-fold at 500 μg/ml, and no transformants were obtained at 1 mg/ml. E. coli TBI carrying each of the four trfA mutant banks was then transformed with pSV16 and selected on 750 μg of penicillin per ml. Samples of each transformation were also plated on 50 μg of kanamycin per ml to determine the number of total transformants capable of supporting pSV16. Of the total transformants, 0.4% from the pRD110-16 20-min bank, 1.1% from the pRD110-16 40-min bank, 4.0% from the pRD110-34 20-min bank, and 10.6% from the pRD110-34 40-min bank were resistant to 750 μg of penicillin per ml. A control transformation of cells containing unmutagenized pRD110-34 yielded no colonies on 750 μg of penicillin per ml (less than 0.1%).

Individual colonies growing on plates containing 750 μg of penicillin per ml were tested to confirm that they were in fact resistant to higher levels of penicillin than colonies containing pSV16 and unmutagenized pRD110-16 or pRD110-34. This was found to be the case for at least 90% of the colonies examined. Plasmid DNA was isolated from 44 such colonies. Agarose gel electrophoresis indicated that four colonies contained plasmids of an anomalous size, and these were discarded. The remaining 40 colonies contained two plasmids identical in size to pSV16 and either pRD110-16 or pRD110-34, as expected. In at least 70% of these colonies, the amount of pSV16 DNA relative to pRD110 DNA was clearly greater than in the unmutagenized situation, consistent with the interpretation that the high penicillin resistance was due to increased pSV16 copy number. The pRD110 plasmids from six such colonies were introduced into E. coli HB101 and retransformed with pSV16 to confirm that the mutant phenotype was associated with the pRD110 plasmid. Two of these plasmids, derived from the 20-min mutagenesis pool of pRD110-16, were designated pRD110-16cop271C and pRD110-16cop254D. Three were from the 20-min pool of pRD110-34 and were designated pRD110-34cop273C, pRD110-34cop267L, and pRD110-34cop251M. The final mutant, designated pRD110-34cop250V, was from the 40-min pool.

Properties of trfA copy-up mutants. To facilitate subsequent experiments, the tet gene of pRD110-16, pRD110-34, and the six mutant plasmids was in each case inactivated by the insertion of a promoterless cat gene, yielding the chloramphenicol-resistant derivatives pRD119-16, pRD119-34, pRD119-16cop271C, pRD119-16cop254D, pRD119-34cop273C, pRD119-34cop267L, pRD119-34cop251M, and pRD119-34cop250V, respectively (Fig. 1). The relative amounts of pSV16 in the presence of each of these eight plasmids in E. coli HB101 are shown in Fig. 3. The copy number of pSV16 relative to that of pRD119 was clearly elevated in the presence of each of the mutants compared to the wild-type situation. In the presence of pRD119-34cop267L or pRD119-34cop250V, the copy number of pSV16 was clearly well above that of the pBR322 replicon present in the pRD119 derivatives.

The absolute copy number of pSV16 in the presence of the two wild-type and the six mutant pRD119 derivatives was determined by using the dot blot assay as described previously (10). To avoid possible cointegration of pSV16 and pRD119 via shared DNA sequences, these and subsequent copy number determinations were performed in the E. coli recA strain HB101. The resulting autoradiogram is shown in Fig. 4. The intensity of each spot was quantified and com-
FIG. 3. Agarose gel analysis of pSV16 copy number in the presence of wild-type and mutant trfA genes. DNA was isolated from E. coli HB101 and digested with EcoRI to linearize each plasmid. Each sample contains pSV16 and the following plasmids: pRD119-16 (wild type) (lane 1), pRD119-16cop271C (lane 2), pRD119-16cop254D (lane 3), pRD119-34 (wild type) (lane 4), pRD119-34cop273C (lane 5), pRD119-34cop267L (lane 6), pRD119-34cop251M (lane 7), and pRD119-34cop250V (lane 8).

FIG. 4. Dot blot analysis of pSV16 copy number in the presence of wild-type and mutant TrfA proteins. Samples in rows A through D contain material from 0.02 OD600 units of E. coli HB101 with the following plasmids. Lanes: 1, none; 2, pRD119-34wt; 3, pRD119-16wt; and pSV16; 4, pRD119-16cop271C and pSV16; 5, pRD119-16cop254D and pSV16; 6, 1:5 dilution of lane 5; 7, pRD119-34wt and pSV16; 8, pRD119-34cop273C and pSV16; 9, pRD119-34cop267L and pSV16; 10, 1:5 dilution of lane 9; 11, pRD119-34cop251M and pSV16; 12, 1:5 dilution of lane 11; 13, pRD119-34cop250V and pSV16; 14, 1:5 dilution of lane 13. The 1:5 dilutions in lanes 6, 10, 12, and 14 were prepared with plasmid-free HB101. In each lane, rows A and B are duplicates of one culture, and rows C and D are duplicates of an independent culture. More than 90% of the cells in cultures with pSV16 contained the plasmid (determined as described in reference 10), with two exceptions. About 70% of HB101 (pRD119-34cop250L) cells and about 30% of HB101 (pRD119-34cop250V) cells had lost pSV16. These values were taken into account in calculating plasmid copy number. Standards (row E) are twofold serial dilutions of purified oriV-containing pTJS65 DNA, with the highest amount being equivalent to 10^9 molecules of plasmid. Hybridization was with the nick translated DraI-BamHI oriV fragment of pTJS65, as previously described (10).

The ability of different amounts of wild-type TrfA proteins to suppress the copy-up phenotype of these mutants in trans was determined by using two plasmids, pAL100 and pAL102. These plasmids are RSF1010 replicons that are compatible with the pBR322 and minimal RK2 origins of replication, pRD119 and pSV16, respectively. They carry the promoterless wild-type trfA gene downstream of a polylinker. In pAL102, a derivative of the R6K pir promoter (35) was inserted into the polylinker of pAL100 to increase the expression level of trfA.

Table 2 lists the copy numbers of pSV16 in the presence of wild-type or mutant pRD119-34 together with either pAL100 or pAL102. In general, wild-type trfA exhibited at least partial dominance over the mutants. Only pRD119-34cop267L and pRD119-34cop273C were fully recessive to pAL100. With the other copy-up derivatives, pAL100 only partially suppressed the copy number of pSV16. However, in the presence of pAL102, pSV16 copy number was reduced to near-normal levels in all cases. A possible explanation for these observations lies in the relative amounts of wild-type and mutant plasmids in each situation. Western analysis indicated that pRD119-34 made about 240 ng of total TrfA per unit of optical density at 600 nm (OD600) (data not shown). The mutant derivatives all made similar amounts (Fig. 5). Previous experiments estimated the total TrfA produced by pAL100 and pAL102 at 28 and 280 ng per OD600 unit, respectively (10). Thus, when mutant trfA is present in substantial excess over wild-type trfA (approximately ninefold), the copy number of pSV16 is generally intermediate between the wild-type and mutant levels. When wild-type and mutant trfA are present in equivalent amounts, the copy number of pSV16 approaches the wild-type level. Additional experiments with other copy-up trfA derivatives suggest that this is a general property of such mutants (R. Durland, unpublished observations). However, it is noteworthy that in some cases, such as pRD119-34cop267L, the mutant is recessive to even low levels of wild-type TrfA proteins provided in trans (i.e., from pAL100).

Mapping and sequencing of the mutations in trfA. The location of the mutation responsible for the copy-up phenotype was mapped in the six mutant plasmids by performing reciprocal exchanges of restriction fragments between individual mutant and wild-type plasmids (described in Materials and Methods). In all six cases, the mutant phenotype was found to be associated with the HindII-to-NdeI fragment of the trfA gene, corresponding to bp 967 to 1346. The sequence of this fragment was determined for each of the six mutated plasmids. Comparison with the wild-type sequence revealed a single G:C to A:T transition in each case (Fig. 6). Each
mutation resulted in a single substitution in the TrfA amino acid sequence, indicating that the mutant phenotype is a result of a change in the TrfA protein(s). All six mutations occurred within a stretch of 69 bp, and with the exception of the cop250V mutation, all resulted in nonconservative amino acid substitutions.

**Effect of copy-up trfA mutations on the replication of RK2 plasmids in trans.** The properties of the replication mutants indicate that TrfA is more than a simple positive factor required for RK2 replication. The above results indicate that at least for a minimal replicon (pSV16), an activity of the TrfA protein(s) is intimately involved in determining replication frequency. Since RK2 carries various other loci (e.g., *kil* and *kor*) that are known or suspected to affect copy number or plasmid maintenance (3, 12, 25, 28, 30–32, 39, 41, 42, 46), we wished to determine how the *trfAcop* mutants interact with intact RK2 and other RK2 derivatives. pRD110-34 and pRD119-34 were used to test the ability of copy-up trfA mutants in trans to increase the copy number of RK2 and a small self-replicating derivative, pFF1 (Fig. 2). The latter plasmid contains oriV and the minimal trfA gene transcribed by the constitutive Tn5 neo promoter (9) but lacks all known *kil* and *kor* genes.

The copy numbers of both pFF1 and RK2 were substantially increased by certain mutant trfA genes in trans (Table 3). In the presence of *trfAcop*250V, the copy number of each plasmid was increased approximately fourfold. Previous results indicate that this is not due to an increase in TrfA concentration per se, since a large excess of wild-type TrfA protein increases RK2 copy number by only 30\% (10). These results demonstrate that certain mutant TrfA proteins are capable of interfering with or partially overcoming the normal replication controls present in RK2. Interestingly, we observed that cells containing RK2 and either pRD119-cop251M or pRD119-34cop250V grew slowly and formed small colonies on LB agar plates. This was not true for RK2 in the presence of pRD119-34 (wild type) or the other two mutants tested, nor was it true in any of the combinations with pFF1, suggesting that it is due to the higher RK2 copy number in the presence of the *cop251M* or *cop250V* mutations. It is possible that the growth inhibition is related to the RK2 *kil* genes, whose host lethality is normally suppressed by the RK2 *kor* genes (3, 12, 25, 28, 31, 47). At elevated RK2 copy numbers, the complex regulatory network involving *kil* and *kor* functions may be perturbed, and partial expression of one or more Kil phenotypes may result in a growth defect.

**DISCUSSION**

This work describes the isolation of mutations in the RK2 trfA gene that increase the copy number of all RK2 replicons tested, including intact RK2. The fact that all six sequenced mutations alter the primary amino acid sequence suggests that the copy-up phenotype is the result of an altered activity of the TrfA protein(s). The possibility that the mutations affect a small regulatory protein whose coding sequence overlaps the trfA gene is small. No single alternative reading frame encompasses the mutated region in such a way that all

### TABLE 2. Copy number of pSV16 in the presence of wild-type and mutant TrfA proteins

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</tr>
<tr>
<td>pRD119-34</td>
<td>Wild type</td>
<td>5.5 ± 1 (1)</td>
<td>5.1 ± 0.3 (1)</td>
<td>5.2 ± 0.4 (1)</td>
</tr>
<tr>
<td></td>
<td>cop273C</td>
<td>16 ± 1 (3)</td>
<td>6.8 ± 0.6 (1.3)</td>
<td>6.3 ± 0.4 (1.2)</td>
</tr>
<tr>
<td></td>
<td>cop267L</td>
<td>120 ± 6 (23)</td>
<td>8.3 ± 0.5 (1.6)</td>
<td>6.4 ± 0.9 (1.2)</td>
</tr>
<tr>
<td></td>
<td>cop251M</td>
<td>32 ± 1 (6)</td>
<td>14 ± 1 (2.7)</td>
<td>8.7 ± 0.5 (1.7)</td>
</tr>
<tr>
<td></td>
<td>cop250V</td>
<td>120 ± 3 (23)</td>
<td>79 ± 2 (15)</td>
<td>13 ± 1 (2.5)</td>
</tr>
</tbody>
</table>

* Copy number was determined in logarithmically growing *E. coli* HB101 as described in the accompanying paper (10). Units are 10^9 molecules per OD_{600} unit (mean ± standard deviation). Numbers in parentheses are approximate relative copy numbers, with the average wild-type value (−5.2 ± 10^6 per OD_{600} unit) taken to be 1. Total TrfA levels are given for each plasmid. Numbers in parentheses (10). One OD_{600} unit is equivalent to ~3.7 × 10^9 cells (detected with a Petroff-Hauser chamber).

* NT, Not tested.

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**FIG. 5.** Anti-TrfA Western blot of *E. coli* HB101 (0.5 OD_{600} units per lane) containing the following plasmids. Lanes: a and b, none; c and d, pRD119-34wt; e and f, pRD119-16wt; g and h, pRD119-16cop271C; i and j, pRD119-16cop254D; k and l, pRD119-34wt; m and n, pRD119-34cop273C; o and p, pRD119-34cop267L; q and r, pRD119-34cop251M; s and t, pRD119-34cop250V. Samples in lanes e through t also contained pSV16.
**FIG. 6.** Nucleotide sequence changes in six copy-up *trfA* mutations. The upper line represents the coding sequence for the *trfA* gene, indicating the two start codons and the stop codon. All copy-up mutations map to the region between the *HincII* and *Ndel* sites. The region containing the sequenced mutations is expanded below. The sense strand of the DNA is numbered according to the system of Smith and Thomas (32). Amino acid residues are numbered from the first methionine of *TrfA*-43.

six sequence changes lead to amino acid substitutions (32). It is formally possible that the mutations affect a *trans*-acting regulatory RNA molecule. However, studies on the RK2 system to date have not suggested the existence of any such RNA molecule specified by the *trfA* gene. Thus, we consider that these mutations define a region of the *TrfA* protein(s) that is intimately involved in RK2 replication initiation and its control. The fact that mutations with similar phenotypes are found regardless of the presence or absence of *TrfA*-43 suggests that the function of the larger protein is not qualitatively different from that of the smaller one in this regard.

Although little is known about the activities of the *TrfA* proteins, it has been observed that one or both proteins bind to repeated sequences (iterons) present in the *oriV* region.

**TABLE 3.** Copy number of pFF1 and RK2 in the presence of excess wild-type or mutant *TrfA* proteins

<table>
<thead>
<tr>
<th><em>trfA</em> allele in <em>trans</em></th>
<th>Plasmid copy number* with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pFF1</td>
</tr>
<tr>
<td>Wild type</td>
<td>16 ± 0.8 (1)</td>
</tr>
<tr>
<td><em>cop273C</em></td>
<td>21 ± 1 (1.3)</td>
</tr>
<tr>
<td><em>cop267L</em></td>
<td>18 ± 1 (1)</td>
</tr>
<tr>
<td><em>cop251M</em></td>
<td>50 ± 3 (3)</td>
</tr>
<tr>
<td><em>cop250V</em></td>
<td>69 ± 5 (4.3)</td>
</tr>
</tbody>
</table>

* Additional TrfA was provided by pRD110-34 (for experiments with pFF1) or pRD119-34 (for experiments with RK2), which differ only in antibiotic resistance genes.

* Copy number was determined in logarithmically growing *E. coli* HB101 as described in the accompanying paper (10). Units are 10⁶ molecules per OD₆₀₀ unit (mean ± standard deviation). Numbers in parentheses are relative copy number, with the wild-type situation for each plasmid (pFF1 or RK2) taken to be 1. Total TrfA levels in cells containing the following plasmids were as follows: pRD110-19, ~235 ng per OD₆₀₀ unit; pFF1, ~23 ng per OD₆₀₀ unit; RK2, ~6.4 ng per OD₆₀₀ unit (determined as described in reference 10). One OD₆₀₀ unit is equivalent to ~3.7 × 10⁶ cells (determined with a Petroff-Hauser chamber).
It is most significant that three of four mutants tested increase the copy number of intact RK2 in E. coli when the mutant proteins are provided in trans. In the presence of pRD119-34cop251M and pRD119-34cop250V, the RK2 copy number is increased approximately fourfold. This is well above the maximum RK2 copy number obtained by substantially overproducing the wild-type TrfA proteins (10). These observations support a multi-functional role for TrfA, with one or more domains of the protein acting in a positive sense and at least one involved in the negative control of plasmid RK2 replication. Similar positive and negative activities for a replication initiation protein have been proposed for plasmids R6K (18, 34) and P1 (7, 23). It is not yet clear why different plasmid derivatives of RK2 show different responses to individual copy-up TrfA proteins provided in trans. In particular, pRD119-34cop250V trfA increases the copy number of the origin plasmid pSV16 15-fold in the presence of moderate amounts of wild-type TrfA provided in trans by pAL100. In contrast, pFF1 and RK2 copy numbers increase only fourfold in the presence of pRD119-34cop250V. Since pFF1 and RK2 each produce wild-type TrfA at levels similar to or substantially less than that specified by pAL100, it seems likely that other elements present on pFF1 and RK2 are modulating the copy-up effect of the TrfA cop250V mutant protein.

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LITERATURE CITED


41. Thomas, C. M., and A. A. K. Hussain. 1984. The korB gene of broad host range plasmid RK2 is a major copy number control element which may act together with trfB by limiting trfA expression. EMBO J. 3:1513–1519.